SPECIFICATION

BIOLOGICALLY NON-DEGRADABLE PEPTIDES, ANGIOTENSIN CONVERTING ENZYME INHIBITOR, DRUG AND FUNCTIONAL FOOD

FIELD OF ART

The present invention relates to novel *in vivo* indigestible peptides that are highly absorbable and hardly digestible in living organism when administered orally or through other route, and to angiotensin converting enzyme inhibitors containing the peptides as active ingredients, and medicine and functional foods, such as foods for specified health use, containing the inhibitor and having hypotensive effect.

BACKGROUND ART

A variety of peptides have been reported having various functions, such as angiotensin converting enzyme (abbreviated as ACE hereinbelow) inhibitory activity, hypotensive effect, anti-bacterial activity, calcium solubilizing effect, and immunomodulating effect, and these peptides are in use in food and medicine.

ACE converts a precursor, angiotensin I, to angiotensin II having vasoconstrictive activity in living organism, to thereby raise the blood pressure. Thus peptides having ACE inhibitory activity are expected to exhibit hypotensive effect by inhibiting ACE to suppress production of angiotensin II in living organism. Such peptides having

ACE inhibitory activity have been reported in many publications including the following Patent Publications 1 to 3.

As peptides that are absorbed through the alimentary canal into blood to express their functions in living organism, *in vivo* indigestible peptides are expected to be advantageous that have high absorbability and digestion resistance against various digestive enzymes in living organism. For example, Patent Publication 4 teaches that low molecular weight peptides mainly composed of dipeptides and tripeptides and having an average chain length of not longer than 3, have excellent intestinal absorbability. However, it is not known in detail which peptides contribute to enhancement of the *in vivo* digestion resistance.

Thus development of peptides are desired that have high absorbability and digestion resistance in living organism, and are capable of effectively exhibiting useful functions, such as ACE inhibitory activity, in living organism.

Patent Publication 1: JP-2-62828-A

Patent Publication 2: JP-3-120225-A

Patent Publication 3: JP-6-40944-A

Patent Publication 4: JP-5-252979-A

SUMMARY OF THE INVENTION

It is an object of the present invention to provide novel *in vivo* indigestible peptides that are highly absorbable and hardly digestible in living organism when administered orally or through other route, and expected

to effectively exhibit functions such as hypotensive effect in living organism.

It is another object of the present invention to provide an angiotensin converting enzyme inhibitor containing, as active ingredients, *in vivo* indigestible peptides that are highly absorbable and hardly digestible in living organism, and effectively exhibiting angiotensin I converting enzyme inhibitory activity in living organism.

It is still another object of the present invention to provide medicine or functional food containing, as active ingredients, *in vivo* indigestible peptides that are highly absorbable and hardly digestible in living organism, and effectively exhibiting hypotensive effect in living organism.

The present inventors have made intensive researches to find out that, among dipeptides and tripeptides having good *in vivo* absorbability, peptides having dipeptide Xaa-Pro or tripeptide Xaa-Pro-Pro sequences (Xaa may be any amino acid) with Pro at the carboxyl terminals, are indigestible in living organism, and capable of effectively exhibiting their functions in living organism. The inventors have also found out that, among the dipeptides and tripeptides having such sequences, those having particular sequences are particularly excellent in ACE inhibitory activity or the like properties, to thereby complete the present invention.

According to the present invention, there is provided

an *in vivo* indigestible peptide having Pro at a carboxyl terminal, selected from the group consisting of Ile-Pro, Glu-Pro, Arg-Pro, Gln-Pro, Met-Pro, and Ser-Pro-Pro.

According to the present invention, there is also provided an ACE inhibitor comprising as active ingredients *in vivo* indigestible peptides having Pro at carboxyl terminals, consisting at least one of Ile-Pro, Glu-Pro, Arg-Pro, Gln-Pro, Met-Pro, and Ser-Pro-Pro, or a salt thereof.

According to the present invention, there is further provided a medicine or functional food having hypotensive effect comprising the ACE inhibitor.

According to the present invention, there is also provided use of the ACE inhibitor in the manufacture of functional food or medicine having hypotensive effect.

Since the *in vivo* indigestible peptides of the present invention are dipeptides or tripeptides of particular sequences with Pro at the carboxyl terminals, the present peptides are highly absorbable and hardly digestible in living organism, and expected to effectively exhibit functions, such as hypotensive effect, in living organism.

Since the ACE inhibitor of the present invention contains the above *in vivo* indigestible peptides as active ingredients, the present inhibitor is expected to effectively exhibit ACE inhibitory activity in living organism.

Since the medicine and functional food of the present

invention contain the ACE inhibitor, the present medicine and functional food are expected to effectively exhibit hypotensive effect in living organism.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a graph showing the results of evaluation of *in vivo* absorbability and digestion resistance of Xaa-Pro and Xaa-Pro-Pro performed in Referential Example 1.

PREFERRED EMBODIMENTS OF THE INVENTION

The present invention will now be explained in detail.

The *in vivo* indigestible peptide of the present invention is a dipeptide or tripeptide having Pro at the carboxyl terminal, consisting at least one of Ile-Pro, Glu-Pro, Arg-Pro, Gln-Pro, Met-Pro, and Ser-Pro-Pro.

As used herein, the *in vivo* indigestible peptide means a dipeptide Xaa-Pro or a tripeptide Xaa-Pro-Pro having Pro at the carboxyl terminal, which has high digestion resistance against *in vivo* peptidases when absorbed intestinally in living organism.

The *in vivo* indigestible peptide of the present invention may be prepared, for example, from the corresponding amino acids by ordinary organic synthesis or the like method. Alternatively, the present peptide may also be prepared, for example, by digesting food protein, such as animal milk casein, through fermentation with lactic acid bacteria, followed by purification; or by hydrolyzing food protein through an enzymatic method with an appropriate combination of proteinases and peptidases, followed by

purification.

The purification does not have to result in isolation of the dipeptide or tripeptide, and may be concentration and purification by a suitable combination of conventional purification methods for increasing the peptide concentration.

The animal milk casein may be, for example, casein from cow's milk, horse's milk, goat's milk, and sheep's milk, with cow's milk casein being particularly preferred.

The ACE inhibitor of the present invention contains, as active ingredients, *in vivo* indigestible peptides having Pro at the carboxyl terminals, consisting at least one of Ile-Pro, Glu-Pro, Arg-Pro, Gln-Pro, Met-Pro, and Ser-Pro-Pro, i.e., the *in vivo* indigestible peptides of the present invention, or a salt thereof.

The peptide salt is preferably a pharmaceutically acceptable salt, such as salts of inorganic acid including hydrochloride, sulfate, or phosphate, or of organic acid including acetate, trifluoroacetate, citrate, maleate, fumarate, lactate, or tartrate.

The ACE inhibitor of the present invention may optionally contain various auxiliary additives for improving the nutritional balance or flavor. Examples of such auxiliary additives may include various carbohydrates, lipids, vitamins, minerals, sweeteners, flavoring agents, pigments, and texture improvers.

The amount of the ACE inhibitor for use may suitably

be selected depending on the kinds of peptides and salts thereof contained as active ingredients, and is not particularly limited. For example, when the ACE inhibitor is added to functional food or the like for regular use by human, the amount of the inhibitor in terms of peptides may preferably be about 0.01 to 100 mg/kg per ingestion, with no particular upper limit.

For use in medicine having hypotensive effect, the ACE inhibitor of the present invention may be formulated with a pharmaceutically acceptable carrier into various dosage forms in accordance with conventional methods. For example, for solid formulation for oral administration, the present inhibitor may be mixed with a vehicle, and optionally a binder, disintegrator, lubricant, coloring agent, taste corrigent, flavor corrigent, or the like as desired, and formulated into tablets, coated tablets, granules, powders, capsules, or the like.

Such medicine may optionally contain other components such as peptides having ACE inhibitory activity or hypotensive effect other than the active ingredients of the present ACE inhibitor.

The ACE inhibitor of the present invention may be used by mixing in functional food such as foods for specified health use claiming hypotensive effect. Examples of such functional food may include beverages, yogurt, liquid food, jelly, candies, retort food, tablet candies, cookies, sponge cakes, bread, biscuits, and chocolates. The

inhibitor may also be formulated into capsules or tablets for use as dietary supplements.

EXAMPLES

The present invention will now be explained in more detail with reference to Example, which is illustrative only and does not intend to limit the present invention.

Example 1

Among peptides having the sequence Xaa-Pro or Xaa-Pro-Pro present in casein derived from cow's milk, Ile-Pro, Glu-Pro, Arg-Pro, Gln-Pro, Met-Pro, and Ser-Pro-Pro were chemically synthesized (manufactured by TORAY RESEARCH CENTER, INC., not lower than 95 % purity). Solutions of each of these peptides at various concentrations were prepared, and measured for the ACE inhibitory activity in accordance with the method discussed below. The peptide concentration (μM) at which the ACE inhibitory effect was 50% was taken as IC50 value. The results are shown in Table 1.

<Evaluation of ACE Inhibitory Activity>

ACE derived from bovine lung (manufactured by WAKO PURE CHEMICAL INDUSTRIES, LTD.) was dissolved in a 0.1M borate buffer at pH 8.3 in an amount of 0.1 U, to obtain an ACE solution. 80 μl of a diluted solution prepared by properly diluting a 5 mg/ml solution of each peptide shown in Table 1 with distilled water according to the IC50 value of that peptide, 200 μl of a 5 mM hippuryl-histidyl-leucine (manufactured by SIGMA) solution containing 300 mM NaCl,

and 20 μl of the ACE solution prepared above were introduced into a tube, and reacted at 37 °C for 30 minutes. Subsequently, the reaction was terminated by adding 250 μl of 1N hydrochloric acid (manufactured by WAKO PURE CHEMICAL INDUSTRIES, LTD.). 1.7 ml of ethyl acetate (manufactured by WAKO PURE CHEMICAL INDUSTRIES, LTD.) was then added, and stirred. 1.4 ml of the ethyl acetate layer was taken, placed in another tube, and evaporated at 120 °C for about 60 minutes to obtain a dried product. The dried product was dissolved in 1 ml of distilled water, and the absorbance at 228 nm of hippuric acid extracted with ethyl acetate was measured. As controls, absorbance was measured of a solution without the diluted solution and a solution without the diluted solution and the ACE solution. From the obtained absorbance, ACE inhibitory activity was calculated in accordance with the following formula.

ACE inhibitory activity (%) = [(A-B)/A] × 100

A: (Absorbance of solution without diluted solution but with ACE solution) - (Absorbance of solution without diluted solution and ACE solution)

B: (Absorbance of solution with diluted solution and ACE solution) - (Absorbance of solution with diluted solution but without ACE solution)

Table 1

Peptide sequence	IC50 value (μM)
Ile-Pro	443.9
Glu-Pro	174.7
Arg-Pro	275.2
Gln-Pro	65.8
Met-Pro	135.3
Ser-Pro-Pro	44.5

Referential Example 1

<Evaluation of Absorbability and Digestion Resistance of Xaa-Pro and Xaa-Pro-Pro in Living Organism>

In order to evaluate the absorbability and digestion resistance in living organism of the peptides having the sequence Xaa-Pro or Xaa-Pro-Pro present in casein, the absorption of the peptides into blood after oral administration was tested in rats as follows.

To two six-week-old SD (Sprague Dawley) rats, 500 mg/animal each of Val-Pro-Pro as an example of Xaa-Pro-Pro and Gly-Gly as a dipeptide having no Pro were administered orally. Blood samples were taken from the portal vein at intervals, and the absorption of each peptide into blood was determined. The results are shown in Fig. 1.

From Fig. 1, it was confirmed that Gly-Gly was easily digested *in vivo*, so that Gly was detected, while Val-Pro-Pro was absorbed into blood relatively stably. From these results, it is expected that the dipeptides and tripeptides having the sequences Xaa-Pro and Xaa-Pro-Pro, respectively, exhibit high absorbability and digestion resistance in

living organism. Thus it is assumed that Ile-Pro, Glu-Pro, Arg-Pro, Gln-Pro, Met-Pro, and Ser-Pro-Pro of the present invention also have high absorbability and digestion resistance in living organism.